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Pulmonary Tuberculosis Conversion Documented by Microscopic Staining for Detection of Dynamic, Dormant, and Dead Mycobacteria (DDD Staining)

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Detecting the infectiousness of *Mycobacterium tuberculosis* mycobacteria in the sputum specimens collected from patients with pulmonary tuberculosis remains cumbersome. Sputum may contain contagious dormant and dynamic mycobacteria along with noncontagious, dead mycobacteria (1, 2, 3). Routine microscopy after auramine or Ziehl-Neelsen staining misses dormant mycobacteria (4, 5), whose detection requires a specific microscopy protocol (5, 6). Additional specific protocols are required for detecting live/dead (7) or metabolically active/inactive *M. tuberculosis* mycobacteria (1). As reported here, we have set up a protocol for the one-shot microscopy detection of dynamic, dormant, and dead mycobacteria—hence, the acronym "DDD" that we gave to this protocol. The DDD protocol incorporates 4′,6-diamidino-2-phenylindole (DAPI) staining of all mycobacteria, fluorescein-di-acetate (FDA) staining of dynamic mycobacteria (8, 9, 10, 11), and Nile red (NR) staining of dormant mycobacteria (see the supplemental material) (5, 6).

As a proof of concept, the DDD protocol was used to monitor the changes in *M. tuberculosis* mycobacteria in sputum specimens collected from one patient treated for pulmonary tuberculosis. A 33-year-old woman with a medical history of community-acquired pneumonia at the age of 15 years was diagnosed in August 2017 with pulmonary tuberculosis. Acid-fast bacilli observed in a sputum smear after Ziehl-Neelsen staining, identified as rifampin-susceptible *M. tuberculosis* by real-time PCR, grew in a solid culture medium (12). The patient benefited from standard antituberculous treatment associating oral ethambutol, pyrazinamide, isoniazid, and rifampin. The clinical course was favorable and rifampin blood dosages confirmed the patient's adherence to therapy (serum peak concentration, 8 μg/ml), but continuous observation of acid-fast bacilli over 6 weeks brought into question the contagiousness of the patient.

Of 133 mycobacteria observed by DDD staining in three Ziehl-Neelsen-positive, culture-positive sputum specimens collected before treatment, 92 (69.2%) were dynamic mycobacteria, 33 (24.8%) were dead mycobacteria, and 8 (6%) were dormant mycobacteria, staining DAPI positive and either FDA positive or FDA negative (Fig. 1A to C). Of 159 mycobacteria observed by DDD staining in three Ziehl-Neelsen-positive, culture-negative sputum specimens collected 24 to 60 days after the initiation of the antituberculous treatment, 47 (29%) were dynamic, 88 (55.5%) dead, and 24 (15.5%) dormant, the cell lengths of which were up to 4.4 μm (Fig. 1A and D). No bacilli were observed after DDD staining of one negative-control decontaminated sputum sample.

The significant decrease in mycobacterial vitality measured by DDD staining after treatment ($P < 0.001$) correlated with a favorable clinical course and sputum conver-

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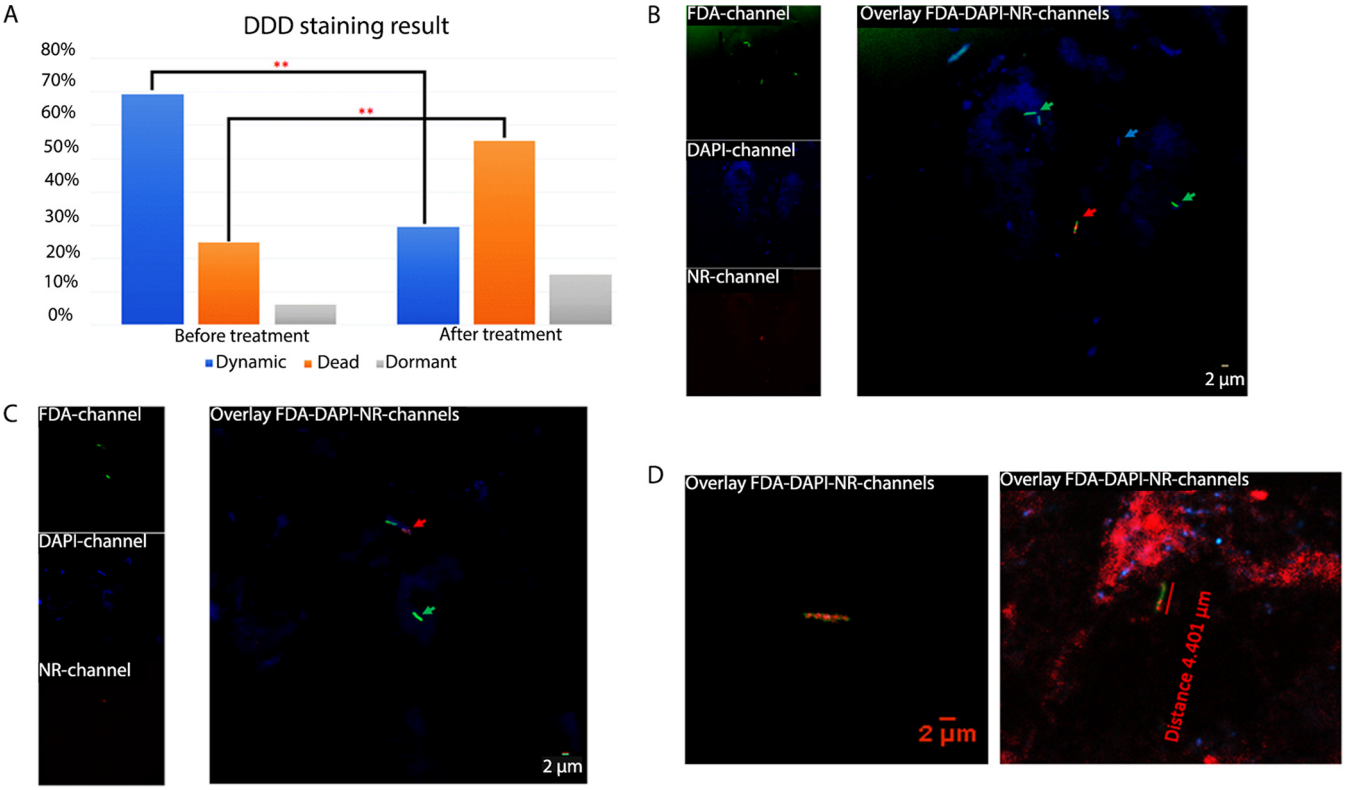

FIG 1 DDD staining results after microscopic observation and counting. DAPI, FDA, and NR channels were used for the microscopic acquisition and image overlay. Image acquisition was performed with a Zeiss spinning-disk confocal microscope using a 63×, 1.4 numeric aperture (NA) oil immersion objective. (A) Comparison between the percentages of dynamic, dead, and dormant *Mycobacterium tuberculosis* mycobacteria in the sputum specimens collected before and after antituberculous treatment. **, $P < 0.001$ by Pearson's chi-square statistic test. (B) Fluorescence microscopy images of sputum smears collected before antituberculous treatment showing dynamic mycobacteria as FDA- and DAPI-positive bacilli (green arrows), dead mycobacteria as DAPI-positive and FDA-negative bacilli (blue arrow), and dormant mycobacterium as FDA- and DAPI-positive bacilli holding NR-positive intracellular inclusions (red arrow). (C) Fluorescence microscopy images of sputum smears from specimens collected before antituberculous treatment, showing dynamic mycobacteria as FDA- and DAPI-positive bacilli (green arrow) and a dormant mycobacterium as FDA-negative and DAPI-positive bacilli with NR-positive intracellular lipid bodies (red arrow). (D) Fluorescence microscopy images of sputum smears from specimens collected after antituberculous treatment, showing a dormant *M. tuberculosis* bacterium holding five NR-positive intracellular inclusions and a dormant *M. tuberculosis* bacterium measuring 4.4 μm and containing 2 lipid bodies.

sion, as previously reported (7, 8). And yet, the percentages of dormant mycobacteria before and after treatment did not differ significantly (Fig. 1A), suggesting that dormant mycobacteria are not susceptible to antituberculous treatment, in line with a previous observation that an increasing percentage of lipidic-inclusion-positive bacilli in sputum after three to four weeks of antituberculous treatment correlated with an unfavorable outcome (13).

The DDD protocol could be applied to paired sputum specimens collected from patients responding poorly to the antituberculous treatment to precisely determine the infectiousness of such patients. The DDD protocol can be used on fresh and −80°C-stored sputum specimens that are alcohol inactivated after DDD staining to protect laboratory workers from exposure to viable mycobacteria. From this perspective, we are now considering automatizing the DDD detection by either using a recently developed scanner protocol or coupling it with flow cytometry (14).

This study was approved by the Ethics Committee of Institut Fédératif de Recherche 48, Marseille, France.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.01108-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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REFERENCES

1. Datta S, Sherman JM, Tovar MA, Bravard MA, Valencia T, Montoya R, Quino W, D'Arcy N, Ramos ES, Gilman RH, Evans CA. 2017. Sputum microscopy with fluorescein diacetate predicts tuberculosis infectiousness. J Infect Dis 216:514–524. https://doi.org/10.1093/infdis/jix229.
2. Dietrich J, Roy S, Rosenkrands I, Lindenstrøm T, Filskov J, Rasmussen EM, Cassidy J, Andersen P. 2015. Differential influence of nutrient-starved *Mycobacterium tuberculosis* on adaptive immunity results in progressive tuberculosis disease and pathology. Infect Immun 83:4731–4739. https://doi.org/10.1128/IAI.01055-15.
3. Woolhiser L, Tamayo MH, Wang B, Gruppo V, Belisle JT, Lenaerts AJ, Basaraba RJ, Orme IM. 2007. *In vivo* adaptation of the Wayne model of latent tuberculosis. Infect Immun 75:2621–2625. https://doi.org/10.1128/IAI.00918-06.
4. Vilchèze C, Kremer L. 2017. Acid-fast positive and acid-fast negative *Mycobacterium tuberculosis*: the Koch paradox. Microbiol Spectr 5(2):TBTB2-0003-2015. https://doi.org/10.1128/microbiolspec.TBTB2-0003-2015.
5. Deb C, Lee C-M, Dubey VS, Daniel J, Abomoelak B, Sirakova TD, Pawar S, Rogers L, Kolattukudy PE. 2009. A novel *in vitro* multiple-stress dormancy model for *Mycobacterium tuberculosis* generates a lipid-loaded, drug-tolerant, dormant pathogen. PLoS One 4:e6077. https://doi.org/10.1371/journal.pone.0006077.
6. Garton NJ, Waddell SJ, Sherratt AL, Lee S-M, Smith RJ, Senner C, Hinds J, Rajakumar K, Adegbola RA, Besra GS, Butcher PD, Barer MR. 2008. Cytological and transcript analyses reveal fat and lazy persister-like bacilli in tuberculous sputum. PLoS Med 5:e75. https://doi.org/10.1371/journal.pmed.0050075.
7. Wyplosz B, Mougari F, Al Rawi M, Baillon C, Marigot-Outtandy D, Le Dû D, Jachym M, Hervé V, Raskine L, Cambau E. 2017. Visualizing viable *Mycobacterium tuberculosis* in sputum to monitor isolation measures. J Infect 74:207–210. https://doi.org/10.1016/j.jinf.2016.11.014.
8. Datta S, Sherman JM, Bravard MA, Valencia T, Gilman RH, Evans CA. 2015. Clinical evaluation of tuberculosis viability microscopy for assessing treatment response. Clin Infect Dis 60:1186–1195. https://doi.org/10.1093/cid/ciu1153.
9. Van Deun A, Maug AKJ, Hossain A, Gumusboga M, de Jong BC. 2012. Fluorescein diacetate vital staining allows earlier diagnosis of rifampicin-resistant tuberculosis. Int J Tuberc Lung Dis 16:1174–1179. https://doi.org/10.5588/ijtld.11.0166.
10. Kvach JT, Veras JR. 1982. A fluorescent staining procedure for determining the viability of mycobacterial cells. Int J Lepr Other Mycobact Dis 50:183–192.
11. Harada S, Numata N. 1992. Application of FDA/EB staining for the detection of viable or non-viable mycobacteria in clinical specimens. Kekkaku 67:113–117. (In Japanese.)
12. Asmar S, Chatellier S, Mirande C, van Belkum A, Canard I, Raoult D, Drancourt M. 2015. A novel solid medium for culturing *Mycobacterium tuberculosis* isolates from clinical specimens. J Clin Microbiol 53:2566–2569. https://doi.org/10.1128/JCM.01149-15.
13. Sloan DJ, Mwandumba HC, Garton NJ, Khoo SH, Butterworth AE, Allain TJ, Heyderman RS, Corbett EL, Barer MR, Davies GR. 2015. Pharmacodynamic modeling of bacillary elimination rates and detection of bacterial lipid bodies in sputum to predict and understand outcomes in treatment of pulmonary tuberculosis. Clin Infect Dis 61:1–8. https://doi.org/10.1093/cid/civ195.
14. DeCoster DJ, Vena RM, Callister SM, Schell RF. 2005. Susceptibility testing of *Mycobacterium tuberculosis*: comparison of the BACTEC TB-460 method and flow cytometric assay with the proportion method. Clin Microbiol Infect 11:372–378. https://doi.org/10.1111/j.1469-0691.2005.01127.x.